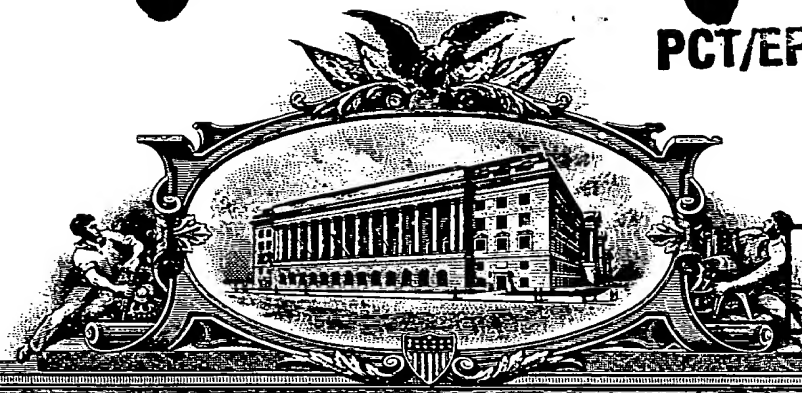
PCT/EP 96/01755



THE UNITED STATES OF AMERICA

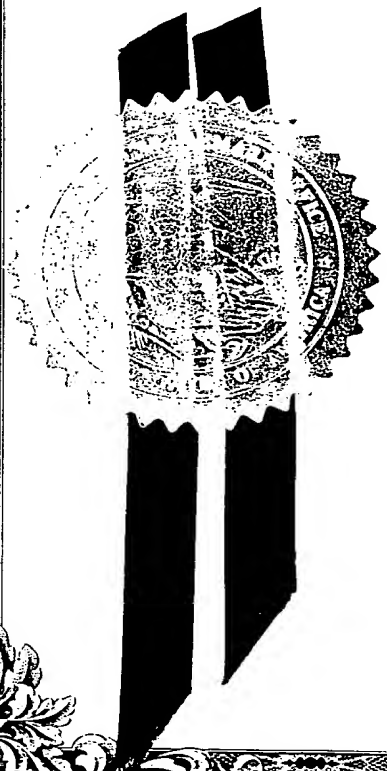
TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office

May 31, 1996

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: ***08/614,115***
FILING DATE: ***March 12, 1996***



PRIORITY DOCUMENT

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

T. Lawrence

T. LAWRENCE
Certifying Officer

PATENT APPLICATION SERIAL NO. 08/614115

U.S. DEPARTMENT OF COMMERCE
PATENT AND TRADEMARK OFFICE
FEE RECORD SHEET

MS18016 04/25/96 08614115 07-1048 180 101 88.00CR

OK refund
88.00
Jac.

PTO-1556
(5/87)

Docket No. GC287
EXPRESS MAIL NO. TB 822 314 196
DATE MAILED: 12-March-1998

NOVEL ALKALINE CELLULASE AND METHOD OF PRODUCING SAME

Piet van Solingen

BACKGROUND OF THE INVENTION

A. Technical field

The present invention relates to novel cellulase compositions. The invention further relates to novel cellulase compositions, preferably derived from *Bacillus sp.* The present invention further relates to the use of the novel cellulase in compositions recognized in the art as advantageously having cellulase added thereto, including, as an additive in a detergent composition, in the treatment of cellulose containing fabrics, in the treatment of pulp and paper and in the treatment of starch for the production of high fructose corn-syrup or ethanol.

B. State of the Art

Cellulases are enzymes which are capable of the hydrolysis of the β-D-glucosidic linkages in celluloses. Cellulolytic enzymes have been traditionally divided into three major classes: endoglucanases, exoglucanases or cellobiohydrolases and β-glucosidases (Knowles, J. et al. (1987), TIBTECH 5, 255-261); and are known to be produced by a large number of bacteria, yeasts and fungi.

Primary among the applications that have been developed for the use of cellulolytic enzymes are those involving degrading (wood)cellulose pulp into sugars for (bio)ethanol production, textile treatments like 'stone washing' and 'biopolishing', and in detergent compositions. Thus, cellulases are known to be useful in detergent compositions for removing dirt, i.e., cleaning. For example, Great Britain Application Nos. 2,075,028, 2,095,275 and 2,094,826 illustrate improved cleaning performance when detergents incorporate cellulase. Additionally, Great Britain Application No. 1,358,599 illustrates the use of cellulase in detergents to reduce the harshness of cotton containing fabrics.

Another useful feature of cellulases in the treatment of textiles is their ability to recondition used fabrics by making their colors more vibrant. For example, repeated washing of cotton containing fabrics results in a greyish cast to the fabric which is believed to be due to disrupted and disordered fibrils, sometimes called "pills", caused by mechanical action. This greyish cast is particularly noticeable on colored fabrics. As a consequence, the ability of cellulase to remove the disordered top layer of the fiber and thus improve the overall appearance of the fabric has been of value.

Despite knowledge in the art related to many cellulase compositions having some or all of the above properties, there is a continued need for new cellulases having a varying spectrum of characteristics which are useful in, for example, treating textiles, as a component of detergent compositions, in the treatment of pulp and paper, and in the conversion of biomass. Applicants have discovered certain cellulases which have such a complement of characteristics and which are useful in such known applications of cellulase.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a novel cellulase having beneficial properties for use in detergents, treating textiles and pulp and paper manufacturing.

According to the present invention, a cellulase is obtainable from or derived from *Bacillus sp.* CBS 669.93, or a derivative of said cellulase. CBS 669.93 is deposited at the Centraalbureau voor Schimmelcultures (CBS), Baarn, Netherlands under accession number CBS 669.93, on December 23, 1993 ("CBS 669.93"). Preferably, the novel cellulase comprises an amino acid sequence according to Figures 2A-2C (SEQ ID NO:1), or a derivative thereof having greater than 58% sequence identity, preferably at least 80% sequence identity and more preferably at least 90% sequence identity thereto. The present invention is also directed to a novel cellulase comprising an amino acid sequence according to Figures 2A-2C (SEQ ID NO:1), or a derivative thereof having greater than 72% sequence similarity, preferably at least 80% sequence similarity and most preferably at least 90% sequence similarity.

According to another embodiment, a composition is provided comprising DNA which encodes an amino acid sequence according to Figures 2A-2C (SEQ ID NO:1), or a derivative thereof having greater than 58% sequence identity, preferably 80% sequence identity and more preferably 90% sequence identity thereto. Alternatively, a composition is provided comprising DNA which encodes an amino acid sequence according to Figures 2A-2C (SEQ ID NO:1), or a derivative thereof having greater than 72% sequence similarity, preferably 80% sequence similarity and more preferably 90% sequence similarity thereto.

According to yet another embodiment of the invention, a method of transforming a suitable microorganism with DNA encoding an amino acid sequence according to the invention is provided.

In an especially preferred embodiment of the present invention, the cellulase is a cellulase derived from *Bacillus sp.* CBS 669.93 having a calculated molecular weight of approximately 63 kD. The approximately 63 kD cellulase has a calculated isoelectric point of about 5 and a pH optimum on CMC of about 6 at 40°C and 60°C.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the pH profile activity of an approximately 63 kD cellulase derived from CBS 669.93 at 40°C and 60°C.

Figures 2A-2C show the DNA sequence (SEQ ID NO:2) and corresponding amino acid sequence (SEQ ID NO:1) of an approximately 63 kD cellulase derived from CBS 669.93.

DETAILED DESCRIPTION OF THE INVENTION

"Derivative" is intended to indicate a protein which is derived from the native protein by addition of one or more amino acids to either or both the C- and N-terminal end of the native protein, substitution of one or more amino acids at one or a number of different sites in the native amino acid sequence, deletion of one or more amino acids at either or both ends of the native protein or at one or more sites in the amino acid sequence, or insertion of one or more amino acids at one or more sites in the native amino acid sequence. The preparation of an enzyme derivative is preferably achieved by modifying a DNA sequence which encodes for the native protein, transformation of that DNA sequence into a suitable host, and expression of the modified DNA sequence to form the derivative enzyme. The derivative of the invention includes peptides comprising altered amino acid sequences in comparison with a precursor enzyme amino acid sequence (e.g., a wild type or native state enzyme according to the present invention) and which peptides retain a characteristic enzyme nature of the precursor enzyme but which have altered properties in some specific aspect. For example, an altered cellulase may have an increased pH optimum or increased temperature resistance but will retain its characteristic cellulolytic activity.

A cellulase is "obtainable from" *Bacillus* 668.93 if such cellulase has an amino acid sequence which corresponds to the amino acid sequence of a cellulase which may be obtained from that organism. Thus cellulase with an identical amino

id sequence to the 63 kD cellulase of the invention derived from a different *Bacillus* would be "obtainable from" *Bacillus* 669.93.

"Host cell" means a cell which has the capacity to act as a host and expression vehicle for a recombinant DNA vector according to the present invention. In a preferred embodiment according to the present invention, "host cell" means the cells of *Bacillus*.

"DNA construct" or "DNA vector" means a nucleotide sequence which comprises one or more DNA fragments encoding any of the novel cellulases or cellulase derivatives described above.

In a preferred embodiment, the cellulase is obtainable from the Centraal Bureau voor Schimmelcultures, Baarn, the Netherlands through microorganism deposition number CBS 669.93 (described in application PCT/EP94/04312), deposited under the Budapest Convention on December 23, 1993. As used herein, the deposited species will be referred to as CBS 669.93. In a more preferred embodiment, the cellulase of the invention is an approximately 63 kD cellulase (calculated on the basis of amino acid sequence of the mature protein) derived from CBS 669.93 (referred to herein as the "63 kD Cellulase"). The approximately 63 kD cellulase has a calculated pI for the mature protein of about 5 and a pH optimum on CMC at 40°C and 60°C of about 6.

The gene encoding the amino acid sequence of the approximately 63 kD cellulase was analyzed by comparison with the accessible sequence data in various libraries (GenBank, Swiss-Prot, EMBL and PIR) using the of CAOS/CAMM Center, University of Nijmegen, Holland. A search of databases for a comparison of the cellulase encoded by the DNA sequence of the present invention with cellulases encoded by published or known cellulase gene sequences revealed that the greatest amount of amino acid identity was found in the cellulase CelB of *Bacillus lautus*.

The approximately 63 kD cellulase was shown to be 58% identical in sequence and 72% similar in sequence using the TFastA program as described by Pearson & Lipman, Proc. Nat. Acad. Sci., vol. 85, pp. 2444-2448 (1988). The TFastA Data Searching Program is commercially available in the Sequence Analysis Software Package Version 6.0 (Genetic Computer Group, Univ. Wisconsin Biotechnology Center, Madison, Wisconsin 53705). The sequence of *Bacillus lautus* is found in Jorgensen et al., Gene, vol. 93, pp. 55-60 (1990). Thus, the present invention encompasses a cellulase which has an amino acid sequence

according to that in Figures 2A-2C (SEQ ID NO:1) or a derivative thereof having greater than 58% sequence identity, preferably greater than 80% sequence identity and most preferably greater than 90% sequence identity thereto. The present invention further encompasses a cellulase which has an amino acid sequence having greater than 72% sequence similarity, preferably greater than 80% sequence similarity and most preferably greater than 90% sequence similarity to the amino acid sequence according to Figures 2A-2C (SEQ ID NO:1).

The present invention also discloses a process for the production of the cellulase. In one embodiment, the cellulase may be produced by cultivating a suitable organism, e.g., *Bacillus sp.* CBS 669.93, under conditions so as to produce the cellulase. Preferably, such conditions include those generally suggested for the cultivation of *Bacillus* to maximize cellulase production and include the use of a cellulose derived substrate as an energy source in combination with necessary salts, ions and other well known ingredients. Generally, the medium used to cultivate the cells may be any conventional medium suitable for growing bacteria. The cells may be cultivated under aerobic conditions in a nutrient medium containing assimilable carbon and nitrogen together with other essential nutrients. Suitable carbon sources are carbohydrates such as sucrose, glucose and starch, or carbohydrate containing materials such as cereal grain, malt, rice and sorghum. The carbohydrate concentration incorporated in the medium may vary widely, e.g., up to 25% and down to 1-5%, but usually 8-10% will be suitable, the percentages being calculated as equivalents of glucose. The nitrogen source in the nutrient medium may be of inorganic and/or organic nature. Suitable inorganic nitrogen sources are nitrates and ammonium salts. Among the organic nitrogen sources used regularly in fermentation processes involving the cultivation of bacteria are soybean meal, cotton seed meal, peanut meal, casein, corn, corn steep liquor, yeast extract, urea and albumin. In addition, the nutrient medium should also contain standard trace substances.

The cellulase may be recovered from the medium by conventional procedures including separating the cells from the medium by centrifugation or filtration, if necessary after disruption of the cells, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt, e.g., ammonium sulfate, followed by purification by a variety of chromatographic procedures, e.g., ion exchange chromatography, affinity chromatography or similar art recognized procedures. For the production of the alkaline cellulase according to the invention,

preferred to cultivate under alkaline conditions using media containing a cellulose based energy source.

Preferably, the cellulase according to the present invention is produced utilizing genetic engineering techniques by transforming a suitable host cell with a gene encoding the cellulase and expressing under conditions appropriate for host cell growth and cellulase expression. As a first step, the chromosomal DNA may be obtained from the donor bacterial strain by the method of Saito and Miura (Saito & Miura, Biochim. Biophys. Acta., vol. 72, pp. 619 (1963)) or by a similar method. Restriction enzyme cleavage of the chromosomal DNA thus obtained gives DNA fragments containing the alkaline cellulase gene. For this purpose, any restriction enzyme may be used provided that it does not cleave the region of said gene. In the alternative, a restriction enzyme may be used which cleaves the gene, using however, a reduced enzyme concentration or incubation time to permit only partial digestion. A preferred restriction endonuclease is *Sau*3A. From the resulting digestion mixture, suitable fragments (4-10kb) may be isolated and used to transform a suitable host cell with a DNA construct, e.g., with a DNA construct including the approximately 9 kb DNA fragment encoding the 63 kD cellulase according to the invention in combination with a suitable vector sequence.

The gene encoding the cellulase of the present invention can be cloned using λ-phage (expression) vectors and *E. coli* host cells. (Alternatively PCR cloning using consensus primers designed on conserved domains may be used). Applicants have discovered that transformation of the gene encoding the cellulase of the present invention and expression in *E. coli* results in an active protein. After a first cloning step in *E. coli*, a cellulase gene according to the present invention can be transferred to a more preferred industrial expression host such as *Bacillus* or *Streptomyces* species, a filamentous fungus such as *Aspergillus* or *Trichoderma*, or a yeast such as *Saccharomyces*. High level expression and secretion obtainable in these host organisms allows accumulation of the cellulase in the fermentation medium from which it can subsequently be recovered.

Preferably, the expression host cell comprises a *Bacillus* sp., more preferably *Bacillus licheniformis* or *Bacillus subtilis*. In an especially preferred embodiment, the transformation host is deleted for protease genes to ensure that the product cellulase is not subject to proteolysis in the fermentation broth or concentrates thereof. A preferred general transformation and expression protocol for protease deleted *Bacillus* strains is provided in Ferrari et al., U.S. Patent No.

4,366, incorporated herein by reference. Transformation and expression in *Aspergillus* is described in, for example, Berka et al., U.S. Patent No. 5,364,770, incorporated herein by reference. A preferred promoter when the transformation host cell is *Bacillus* is the *apr*E promoter.

The instant approximately 63 kD cellulase derived from CBS 669.93 has been shown to be useful in buffer systems comprising glycine, ammonium acetate, borax and/or tris. This cellulase has also been found to be activated on CMC by the presence of magnesium and inhibited by the presence of calcium. A proportion of calcium to magnesium of about 250ppm : 750 ppm has also been found to result in an activity benefit.

According to the present invention, the cellulase compositions described above may be employed in detergent compositions according to art-recognized methods of utilizing cellulases in detergents. The excellent activity of the instant cellulase at alkaline pH should result in the present cellulase being especially useful in high pH detergents.

The invention will be explained in more detail in the following examples which are provided for illustrative purposes and should not to be construed as limitative of the invention.

EXAMPLE 1

Screening And Isolation of Cellulase From Alkaline Soil And Water Samples

Two methods were applied for the isolation of cellulase-producing microorganisms from alkaline soil and water samples. In one method, the soil and water samples were suspended in 0.85% saline solution and directly used in the carboxymethyl cellulose (CMC)-agar diffusion assay for detection of cellulase producing colonies. In a second method, the soil and water samples were enriched for cellulase containing strains by incubation in a cellulose containing liquid minimal medium or GAM-medium for 1 to 3 days at 40°C. Cultures that showed bacterial growth were analyzed for cellulase activity using the CMC-agar diffusion assay for detection of cellulase producing colonies. The CMC-agar diffusion assay and enrichment procedure utilized a minimal medium preparation at a pH of about 9.7 comprising 1% KNO_3, 0.1% yeast extract (Difco), 0.1% KH_2PO_4, 0.02% $MgSO_4.7H_2O$, 1% Na_2CO_3, 4% NaCl and 0.25% CMC (Sigma C-4888). For solidification 1.5% agar was added.

One of two procedures was used for the CMC-agar diffusion assay depending on whether colonies or liquid fractions were tested. For testing colonies, cell suspensions in 0.85% saline solution were plated on CMC-containing minimal medium. After incubation for 1 to 3 days at 40°C, the plates were replica plated and the parent plate was flooded with 0.1% Congo Red for 15 minutes. The plates were destained with 1M NaCl for 30 minutes. The strains that showed a clearing zone around the colony were isolated as potential cellulases producing microorganisms. Liquid fractions were assayed by pipetting 40 µl aliquots of enzyme solution or fermentation broth into wells punched out from a layer of 5 mm of minimal medium in a petri dish. After incubation for 16 hours at 40°C cellulase activity was detected by Congo Red / NaCl treatment. The diameter of the clearing zone is a measure for the CMCase activity.

Strains which showed clearing zones using either of the two screening methods were selected for growing up and isolation of cellulase. The colonies were fermented in 25 millilitre GAM-medium in 100 millilitre shake flasks in an Incubator Shaker (New Brunswick Scientific, Edison, NJ, USA), at 250 r.p.m. at 40°C for 72 hours. CMCase activity was determined in the culture broth at pH 9 and 40°C to verify the presence of cellulase in the fermentation broth. The complex medium (GAM) used for enzyme production consisted of Peptone (Difco) 0.5%, Yeast extract (Difco) 0.5%, Glucose. H_2O 1%, KH_2PO_4 0.1%, $MgSO_4.7H_2O$ 0.02%, Na_2CO_3 1%, NaCl 4%. The pH was adjusted to 9.5 with 4M HCl after which 1% CMC was added.

Utilizing the method described above, a cellulase producing microorganism was isolated which was further characterized as being a motile, long, thin rod-shaped bacterium, occurring in long chains and giving a thread-like appearance or, alternatively, in pairs of cells in a "v" form. The sub-terminal spores were ellipsoidal with a clear swelling of the sporangium. Colonies on GAM-agar appeared as a cream colored, circular, flat, smooth and shiny surfaced with a slightly irregular margin. Based on 16S rRNA sequence analysis, the microorganism was classified as species of the genus *Bacillus*. The organism is referred to herein as CBS 669.93 and is deposited in the Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands under that accession number.

EXAMPLE 2

Isolation of DNA, Transformation and Expression of Cellulase

The alkaliphilic *Bacilli* strain CBS 669.93 was chosen as a donor strain for expression cloning in *E. coli*. Chromosomal DNA was isolated according to the method described by Saito & Miura, Biochim. Biophys. Acta., vol. 72, pp. 619-629 (1963).

The isolated chromosomal DNA was partially digested by the restriction enzyme *Sau*3A using serial diluted enzyme solutions, for one hour at 37°C using React Buffers (Gibco BRL Life Technologies, Gaithersburg, Md., USA) under conditions recommended by the supplier. The digested DNA was fractionated by agarose gel electrophoresis and suitable fractions (4-10 kb) were isolated from the gel using QIAquick Gel Extraction Kit according to the protocol described by the supplier (QIAGEN Inc., Chatsworth, Ca., USA).

The *Sau*3A fragments of the chromosomal DNA were used to construct genomic gene libraries in a *Bam*H1, digested CIAP treated ZAP Express vector according to the protocol described by the supplier (Stratagene Cloning Systems, La Jolla, Ca., USA). pBK-CMV phagmids, containing the cloned DNA inserts, were excised from the ZAP Express™ vector and transformed into *E. coli* strain XLOLR.

Recombinant clones were screened by agar diffusion as described by Wood et al., Meth. Enzym., vol. 160, pp. 59-74 (1988). Strains that showed clearing zones around the colony were isolated. The CMCase activity of the isolated recombinants was determined after fermentation for 48 hours in 4*YEP-medium consisting of Yeast Extract (Difco) 4%, peptone (Difco) 8%, lactose 0.2%, ampicillin 100µg/ml. The recombinant protein was purified (Example 3) and the N-terminal amino acid sequence was determined to be the following:

Asn-Glu-Asp-Val-Lys-Thr-Leu-Asp-Ile-Gln (SEQ ID: NO 3).

Plasmid DNA of the cellulase producing recombinant was isolated using a QIAprep Plasmid Kit according to the protocol described by the supplier (QIAGEN Inc.). The plasmid contained an approximately 9 kb insert of chromosomal DNA. The nucleotide sequence of a fragment of 2777 bp was determined using a set of degenerated oligonucleotides derived from the N-terminal amino acid sequence as a primer to locate the gene on the 9 kb insert. The 2777 bp fragment contained an open reading frame of 1746 bp from which a protein of 574 amino acids could be deduced. The nucleotide sequence of the gene (SEQ. ID. NO 2) coding for said

ce. e and the deduced amino acid sequence (SEQ ID NO 1) of the isolated single cellulase is shown in Figures 2A-2C.

EXAMPLE 3

Purification of Cellulase

The cellulase producing clones from Example 2 were grown on a complex medium (4*YEP) consisting of Yeast Extract (Difco) 4%, Peptone (Difco) 8%, lactose 0.2%, 100 µg/ml ampicillin). The fermentation broth was separated from the culture liquid by centrifugation (8000 rpm). The cellulase in the supernatant was precipitated with ammonium sulphate (65% saturation). The precipitate was dissolved in 25 mM phosphate buffer pH 7 + 5 mM EDTA until a conductivity of 7 mS/cm was achieved. This solution was applied to a Q-Sepharose FF (diameter 5 cm, length 10 cm) Anion Exchange column, after which the column was washed with 25 mM phosphate buffer pH 7 + 5 mM EDTA until an absorbency of 0.2 AU. A gradient of 0 to 0.5 M NaCl in 25 mM phosphate pH 7 was applied to the column in 80 minutes followed by a gradient from 0.5 to 1 M NaCl in 10 minutes. Elution took place in the first gradient. After elution the column was cleaned (upflow) with 1 M NaOH and equilibrated again with 25 mM phosphate pH 7 + 5 mM EDTA. Depending on the elution profile, the obtained cellulase had a purity of up to about 80%.

EXAMPLE 4

Properties of Cellulase According to the Invention

To determine the pH/temperature profile of the approximately 63 kD cellulase according to the invention, the activity of the cellulase was measured on CMC at various pH and temperature values. A solution comprising the approximately 63 kD cellulase was combined in a buffer in diluted with 10 mM phosphate buffer (pH 7). (pH was controlled by using buffer comprising a mixture of 100 ml 1 M phosphoric acid, 100 ml citric acid and 600 ml distilled water having the pH adjusted to 4, 5, 6, 7, 8, 9 or 10 using 4 M NaOH, after which the mixture is filled to 1 L using distilled water). The enzyme solution was diluted until 0.05 U/ml measured at pH 7 and 40°C. Each buffer system was tested to ascertain the actual pH after mixing 0.5 ml Buffer, 0.5 ml substrate (1% CMC) and 0.1 ml 10 mM phosphate buffer. Actual pH for the pH 4, 5, 6, 7, 8, 9 and 10 solutions was 4.2, 5.2, 6.2, 7, 8, 8.7 and 9.9, respectively.

The results are illustrated in Figure 1 showing the excellent alkaline activity of the cellulase. The slope of the calibration curve is dependent on the pH of the enzyme substrate mixture for that reason two glucose standards at each pH are taken (500 mg glucose. H2)/100 ml 10 and 25 times diluted.

Cellulase activity may be assayed using a modified PAHBAH method (Lever M. Anal. Biochem. 1972, 47, 273-279 and Lever M. Anal. Biochem. 1977, 81, 21-27) as follows. The pH/temperature profiles may be determined using a fixed enzyme concentration which fits in the linear range of the dose response profile measured at pH 7 and 40°C. This enzyme concentration may be used for the measurement of the activities under all other determined conditions. A test tube is filled with 250 ml 2.5% CMC in 50 mM glycine buffer pH 9 (CMC-low viscosity is purchased from Sigma) and 250 ml aliquots of the 63 kD cellulase, diluted in the appropriate buffer. The test tube is incubated for 30 minutes at 40°C in a waterbath, whereafter 1.5 ml of a daily fresh prepared PAHBAH solution (1% PAHBAH in 100 ml 0.5 M NaOH with 100 ml bismuth solution (containing 48.5 g bismuth nitrate, 28.2 g potassium sodium tartrate and 12.0 g NaOH in 100 ml) is added. The mixture is heated at 70°C for 10 minutes, after which it is cooled on ice for 2 minutes. The absorption is measured at 410 nm. To eliminate the background absorbance of the enzyme samples a control experiment is executed as follows: a tube with substrate is incubated under the same conditions as the test tube. After the incubation 1.5 ml PAHBAH and the enzyme preparation is added (in this order). One unit (U) is defined as the amount of enzyme producing 1 µmol of glucose from CMC equivalent determined as reducing sugars per minute per gram product.

#3

DECLARATION
AND POWER OF ATTORNEY

DOCKET INFORMATION

ORIGINAL APPLICATION

GC287

AS A BELOW NAMED INVENTOR, I HEREBY DECLARE THAT:

MY RESIDENCE, POST OFFICE ADDRESS AND CITIZENSHIP ARE AS STATED BELOW NEXT TO MY NAME. I BELIEVE I AM THE ORIGINAL, FIRST AND SOLE INVENTOR (IF ONLY ONE NAME IS LISTED BELOW) OR AN ORIGINAL, FIRST AND JOINT INVENTOR (IF PLURAL NAMES ARE LISTED BELOW) OF THE SUBJECT MATTER WHICH IS CLAIMED AND FOR WHICH A PATENT IS SOUGHT ON THE INVENTION ENTITLED **NOVEL ALKALINE CELLULASE AND METHOD OF PRODUCING SAME**

CHECK ONE:

☐ IS ATTACHED HERETO

☒ WAS FILED ON MARCH 12, 1996, AS APPLICATION SERIAL NO. 614,115 AND WAS AMENDED ON.

I HEREBY STATE THAT I HAVE REVIEWED AND UNDERSTAND THE CONTENTS OF THE ABOVE IDENTIFIED SPECIFICATION, INCLUDING THE CLAIMS, AS AMENDED BY ANY AMENDMENT REFERRED TO ABOVE. I ACKNOWLEDGE THE DUTY TO DISCLOSE INFORMATION WHICH IS MATERIAL TO PATENTABILITY AS DEFINED IN TITLE 37, CODE OF FEDERAL REGULATIONS §1.56.

I HEREBY CLAIM FOREIGN PRIORITY BENEFITS UNDER TITLE 35, UNITED STATES CODE §119, OF ANY FOREIGN APPLICATION(S) FOR PATENT OR INVENTOR'S CERTIFICATE LISTED BELOW AND HAVE ALSO IDENTIFIED BELOW ANY FOREIGN APPLICATION FOR PATENT OR INVENTOR'S CERTIFICATE HAVING A FILING DATE BEFORE THAT OF THE APPLICATION ON WHICH PRIORITY IS CLAIMED.

APPLICATION NUMBER	COUNTRY	DATE OF FILING	PRIORITY CLAIMED YES	NO

I HEREBY CLAIM THE BENEFIT UNDER TITLE 35, UNITED STATES CODE §120, OF ANY UNITED STATES APPLICATION(S) LISTED BELOW AND, INSOFAR AS THE SUBJECT MATTER OF EACH OF THE CLAIMS OF THIS APPLICATION IS NOT DISCLOSED IN THE PRIOR UNITED STATES APPLICATION IN THE MANNER PROVIDED BY THE FIRST PARAGRAPH OF TITLE 35, UNITED STATES CODE §112, I ACKNOWLEDGE THE DUTY TO DISCLOSE MATERIAL INFORMATION AS DEFINED IN TITLE 37, CODE OF FEDERAL REGULATIONS §1.56(A) WHICH OCCURRED BETWEEN THE FILING DATE OF THE PRIOR APPLICATION AND THE NATIONAL OR PCT INTERNATIONAL FILING DATE OF THIS APPLICATION.

APPLICATION NUMBER	DATE OF FILING	STATUS - PATENTED, PENDING OR ABANDONED

POWER OF ATTORNEY: AS A NAMED INVENTOR I HEREBY APPOINT AS MY ATTORNEY(S) WITH FULL POWER OF SUBSTITUTION AND REVOCATION, TO PROSECUTE THIS APPLICATION AND TRANSACT ALL BUSINESS IN THE PATENT AND TRADEMARK OFFICE CONNECTED THEREWITH:

MARGARET A. HORN, REG. NO. 33,401
CHRISTOPHER L. STONE, REG. NO. 35,696
KIRSTEN A. ANDERSON, REG. NO. 38,813

SEND CORRESPONDENCE TO:	DIRECT TELEPHONE CALLS TO.
CHRISTOPHER L. STONE GENENCOR INTERNATIONAL, INC. 180 KIMBALL WAY SOUTH SAN FRANCISCO, CA 94080	415/742-7555

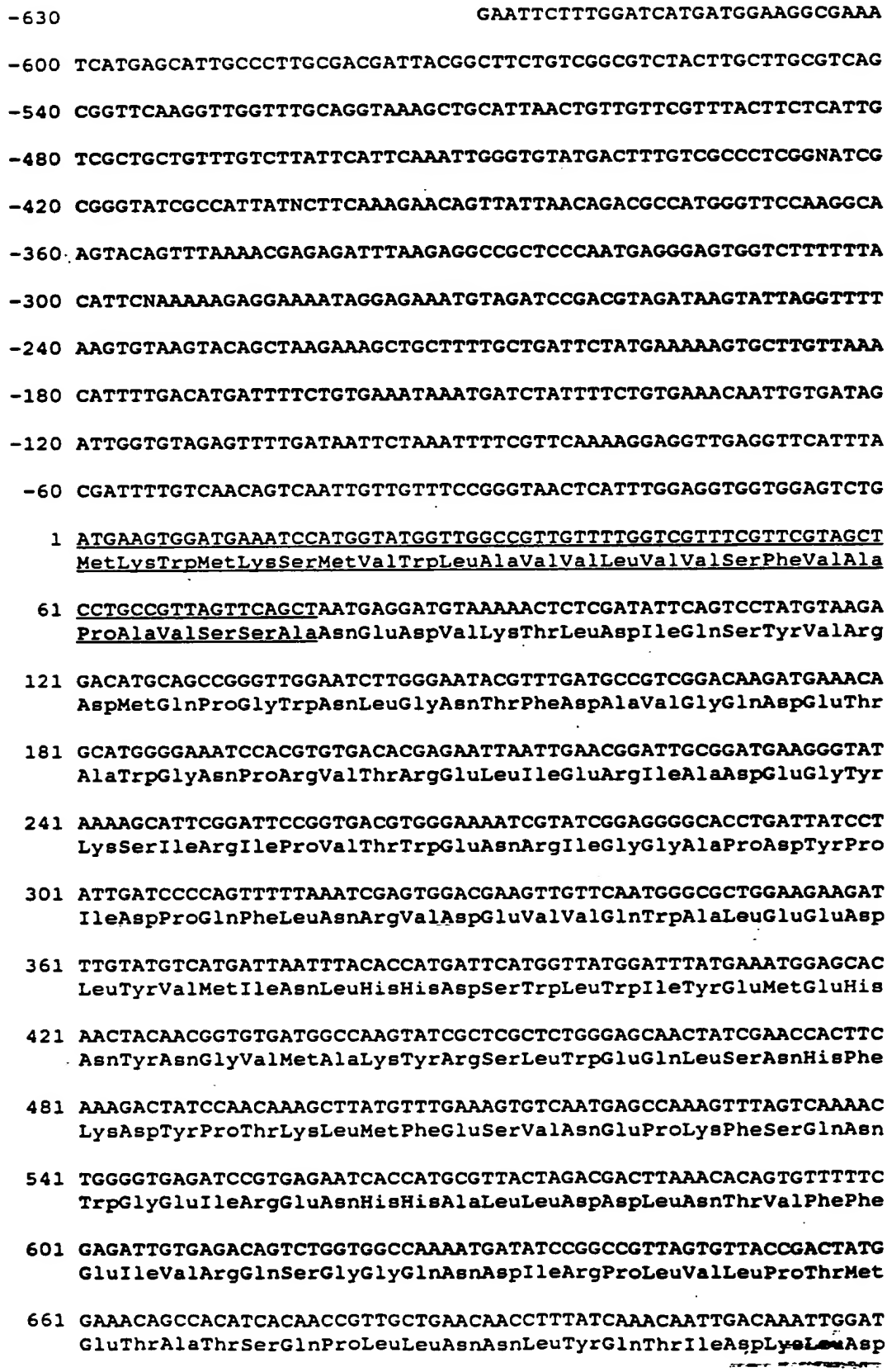
201

FULL NAME OF INVENTOR	FULL FIRST NAME	INITIAL	LAST NAME		
	PIETER (ALSO KNOWN AS PIET)		VAN SOLINGEN		
RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP		
	NAALDWIJK	THE NETHERLANDS	THE NETHERLANDS		
POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE OR COUNTRY	ZIP CODE	
	ROSSINI 16	NAALDWIJK	THE NETHERLANDS	2671 VZ	

I FURTHER DECLARE THAT ALL STATEMENTS MADE HEREIN OF MY OWN KNOWLEDGE ARE TRUE AND THAT ALL STATEMENTS MADE ON INFORMATION AND BELIEF ARE BELIEVED TO BE TRUE; AND FURTHER THAT THESE STATEMENTS WERE MADE WITH THE KNOWLEDGE THAT WILLFUL FALSE STATEMENTS AND THE LIKE SO MADE ARE PUNISHABLE BY FINE OR IMPRISONMENT, OR BOTH, UNDER SECTION 1001 OF TITLE 18 OF THE UNITED STATES CODE , AND THAT SUCH WILLFUL FALSE STATEMENTS MAY JEOPARDIZE THE VALIDITY OF THE APPLICATION OR ANY PATENT ISSUING THEREON.

SIGNATURE OF INVENTOR 201	
DATE	06 May 1996

FIGURE 2A


-630 GAATTCTTTGGATCATGATGGAAGGCGAAA
-600 TCATGAGCATTGCCCTTGCGACGATTACGGCTTCTGTCGGCGTCTACTTGCTTGCGTCAG
-540 CGGTTCAAGGTTGGTTTGCAGGTAAAGCTGCATTAACTGTTGTTCGTTTACTTCTCATTG
-480 TCGCTGCTGTTTGTCTTATTCATTCAAATTGGGTGTATGACTTTGTCGCCCTCGGNATCG
-420 CGGGTATCGCCATTATNCTTCAAAGAACAGTTATTAACAGACGCCATGGGTTCCAAGGCA
-360 AGTACAGTTTAAAACGAGAGATTTAAGAGGCCGCTCCCAATGAGGGAGTGGTCTTTTTTA
-300 CATTCNAAAAAGAGGAAAATAGGAGAAATGTAGATCCGACGTAGATAAGTATTAGGTTTT
-240 AAGTGTAAGTACAGCTAAGAAAGCTGCTTTTGCTGATTCTATGAAAAAGTGCTTGTTAAA
-180 CATTTTGACATGATTTTCTGTGAAATAAATGATCTATTTTCTGTGAAACAATTGTGATAG
-120 ATTGGTGTAGAGTTTTGATAATTCTAAATTTTCGTTCAAAAGGAGGTTGAGGTTCATTTA
-60 CGATTTTGTCAACAGTCAATTGTTGTTTCCGGGTAACTCATTTGGAGGTGGTGGAGTCTG
1 ATGAAGTGGATGAAATCCATGGTATGGTTGGCCGTTGTTTTGGTCGTTTCGTTCGTAGCT
MetLysTrpMetLysSerMetValTrpLeuAlaValValLeuValValSerPheValAla
61 CCTGCCGTTAGTTCAGCTAATGAGGATGTAAAAACTCTCGATATTCAGTCCTATGTAAGA
ProAlaValSerSerAlaAsnGluAspValLysThrLeuAspIleGlnSerTyrValArg
121 GACATGCAGCCGGGTTGGAATCTTGGGAATACGTTTGATGCCGTCGGACAAGATGAAACA
AspMetGlnProGlyTrpAsnLeuGlyAsnThrPheAspAlaValGlyGlnAspGluThr
181 GCATGGGGAAATCCACGTGTGACACGAGAATTAATTGAACGGATTGCGGATGAAGGGTAT
AlaTrpGlyAsnProArgValThrArgGluLeuIleGluArgIleAlaAspGluGlyTyr
241 AAAAGCATTCGGATTCCGGTGACGTGGGAAAATCGTATCGGAGGGGCACCTGATTATCCT
LysSerIleArgIleProValThrTrpGluAsnArgIleGlyGlyAlaProAspTyrPro
301 ATTGATCCCCAGTTTTTAAATCGAGTGGACGAAGTTGTTCAATGGGCGCTGGAAGAAGAT
IleAspProGlnPheLeuAsnArgValAspGluValValGlnTrpAlaLeuGluGluAsp
361 TTGTATGTCATGATTAATTTACACCATGATTCATGGTTATGGATTTATGAAATGGAGCAC
LeuTyrValMetIleAsnLeuHisHisAspSerTrpLeuTrpIleTyrGluMetGluHis
421 AACTACAACGGTGTGATGGCCAAGTATCGCTCGCTCTGGGAGCAACTATCGAACCACTTC
AsnTyrAsnGlyValMetAlaLysTyrArgSerLeuTrpGluGlnLeuSerAsnHisPhe
481 AAAGACTATCCAACAAAGCTTATGTTTGAAAGTGTCAATGAGCCAAAGTTTAGTCAAAAC
LysAspTyrProThrLysLeuMetPheGluSerValAsnGluProLysPheSerGlnAsn
541 TGGGGTGAGATCCGTGAGAATCACCATGCGTTACTAGACGACTTAAACACAGTGTTTTTC
TrpGlyGluIleArgGluAsnHisHisAlaLeuLeuAspAspLeuAsnThrValPhePhe
601 GAGATTGTGAGACAGTCTGGTGGCCAAAATGATATCCGGCCGTTAGTGTTACCGACTATG
GluIleValArgGlnSerGlyGlyGlnAsnAspIleArgProLeuValLeuProThrMet
661 GAAACAGCCACATCACAACCGTTGCTGAACAACCTTTATCAAACAATTGACAAATTGGAT
GluThrAlaThrSerGlnProLeuLeuAsnAsnLeuTyrGlnThrIleAspLysLeuAsp

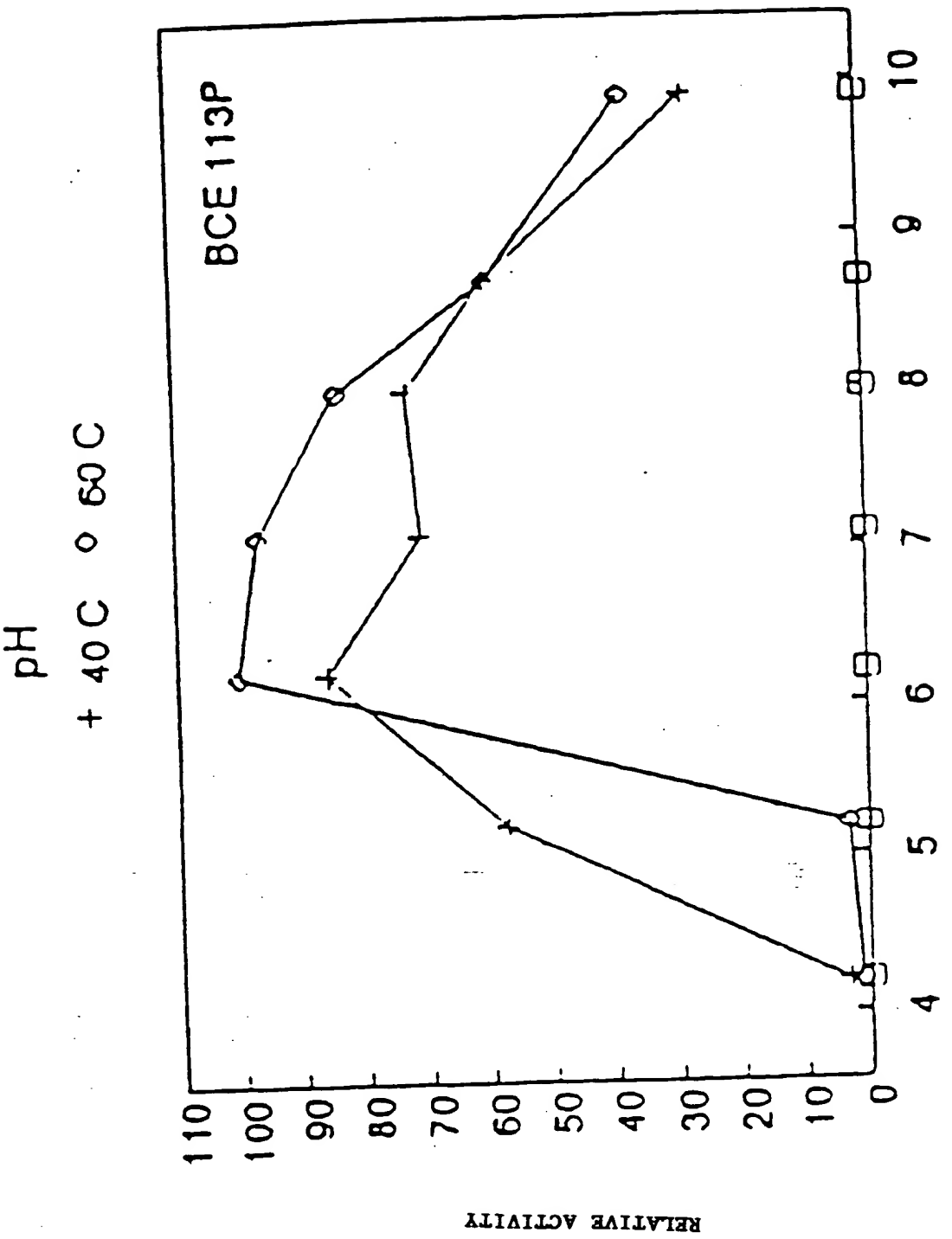

BCE 113P
pH
+ 40 C ○ 60 C
RELATIVE ACTIVITY
110
100
90
80
70
60
50
40
30
20
10
0
4
5
6
7
8
9
10

FIGURE 1

FIGURE 2B

```
721 GATCCGAATCTAATTGCGACAGTACACTATTACGGGTTTTGGCCTTTTAGCGTGAATATC
    AspProAsnLeuIleAlaThrValHisTyrTyrGlyPheTrpProPheSerValAsnIle

781 GCCGGCTACACTCGCTTTGAAGAGGATTCGAAACGGGAGATCATCGAAACGTTTGATCGA
    AlaGlyTyrThrArgPheGluGluAspSerLysArgGluIleIleGluThrPheAspArg

841 GTACACCATACATTTGTTGCAAGAGGGATTCCAGTCGTTTTAGGTGAGTTCGGCTTGCTT
    ValHisHisThrPheValAlaArgGlyIleProValValLeuGlyGluPheGlyLeuLeu

901 GGATTTGATAAACATACTGGAGTGATTCAACAAGGTGAAAAGCTAAAATTCTTTGAGTAT
    GlyPheAspLysHisThrGlyValIleGlnGlnGlyGluLysLeuLysPhePheGluTyr

961 CTCATCCATCATTTGAACGAGCGGGATATTACTCATATGCTTTGGGATAATGGGCAGCAT
    LeuIleHisHisLeuAsnGluArgAspIleThrHisMetLeuTrpAspAsnGlyGlnHis

1021 TTCAATCGTCATACGTACGAATGGTATGACGAGGAATTGTTTGACATGTTGCGGGCAAGC
     PheAsnArgHisThrTyrGluTrpTyrAspGluGluLeuPheAspMetLeuArgAlaSer

1081 TGGGGAGGAAGATCATCCGTTGCAGAGTCGAACTTTATCTATTTAAAACAGGGAGACCGA
     TrpGlyGlyArgSerSerValAlaGluSerAsnPheIleTyrLeuLysGlnGlyAspArg

1141 ATCGCAGATGCAACAGTTACATTACAATTGCACGGAAATGAATTAACAGGGCTTCAGGCG
     IleAlaAspAlaThrValThrLeuGlnLeuHisGlyAsnGluLeuThrGlyLeuGlnAla

1201 AATGGACAACGACTAACGCCGGGGCAGGACTATGAGTTAAATGGAGAAAGACTTACAGTG
     AsnGlyGlnArgLeuThrProGlyGlnAspTyrGluLeuAsnGlyGluArgLeuThrVal

1261 AAGGCCCATGTCCTATCGGCAATCGCAGGTTCAGGTACGTTAGGTACGAATGGAATGGTA
     LysAlaHisValLeuSerAlaIleAlaGlySerGlyThrLeuGlyThrAsnGlyMetVal

1321 ACGGCTGAGTTTAATCGTGGGGCAGATTGGCATTTTCGGGTGAATACGTATCGTACGCCT
     ThrAlaGluPheAsnArgGlyAlaAspTrpHisPheArgValAsnThrTyrArgThrPro

1381 GTATTGCAAAGCACGCAAGGTCACGTGAGCAACTTCAGCATTCCTGCTTCCTTTAATGGG
     ValLeuGlnSerThrGlnGlyHisValSerAsnPheSerIleProAlaSerPheAsnGly

1441 AATAGCTTAGCAACAATGGAGGCTGTCTATGTGGATGGCGGAAATGCTGGCCCGCAAGAC
     AsnSerLeuAlaThrMetGluAlaValTyrValAspGlyGlyAsnAlaGlyProGlnAsp

1501 TGGACCTCCTTTAAGGAGTTTGGCTATGCCTTCTCTCCTTCTTATGATACACATGAGATT
     TrpThrSerPheLysGluPheGlyTyrAlaPheSerProSerTyrAspThrHisGluIle

1561 AAACTGACCGAGGCGTTTTTTCGTGAGGTGCGGGATGGTGAAGTTCGGTTAACCTTCCAT
     LysLeuThrGluAlaPhePheArgGluValArgAspGlyGluValArgLeuThrPheHis

1621 TTTTGGAGTGGTGAAATAGTCAACTATACGATTATTAAAAACGGGAACCAGGTGACTGGG
     PheTrpSerGlyGluIleValAsnTyrThrIleIleLysAsnGlyAsnGlnValThrGly

1681 ATAGCAGCTCAGACAACCAATTCAAAAAACAAAAATAAAAAATGAAATTGAAAGCGCTTT
     IleAlaAlaGlnThrThrAsnSerLysAsnLysAsnLysLysEnd

1741 CTATGGTGTTGCCCGAATATCTGAGGTTCTTTAGTAGAATCCGATATTCGGGTTTTTTCA

1801 TACATTATAGGGGCGCTTTTTTATGTTGCGCAGGTTAAATGGTCTTACGTATGGGAACCC

1861 TACTACTAGATTATTGTGCACTCTTTTTGAGTACCATTATCACCGCCCTATCATATGTAT
```

FIGURE 2C

1921 ATGAGTTGAACCATCTAGTAACCTCTCTTAAAATTGGTAAAGGAAATGTAACGTTGTGAT

2041 AGTAAGGAAATGGTATGATGGAGAGAGACGTGTGATCGAGAAATGGAGGAACGCAGAATG

2101 AATGAAACGATGCAACGCATCGCGAGAGTCATAGAGAATGTGGAACGAGTGGCCGCCGGG

2161 AAACGTCAGGAAATCGAGCTGAGCCTTGTCGCATTATTTGCTAGCGG